Enhancement of Paclitaxel-Mediated Cytotoxicity in Lung Cancer Cells by 17-Allylamino Geldanamycin: In Vitro and In Vivo Analysis

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Background. It has previously been demonstrated that 17-allylamino geldanamycin (17-AAG) enhances paclitaxel-mediated cytotoxicity and downregulates vascular endothelial factor expression in non-small cell lung cancer. This project was designed to evaluate the tumoricidal and antiangiogeneic effects of 17-AAG and paclitaxel in H358 non-small cell lung cancer cells grown as xenografts in nude mice.

Methods. In vitro cytotoxic drug combination effects were evaluated by (4, 5-dimethylthiazo-2-yl)-2, 5-diphenyl tetrazolium bromide-based proliferation assays. The combinations of 17-AAG and paclitaxel were administered intraperitoneally in nude mice bearing H358 tumor xenografts. Tumor volumes were measured weekly. Tumor expression of erbB2, vascular endothelial cell growth factor, von Willebrand factor (tumor microvasculature), and activated caspase 3 (apoptosis) were determined by immunohistochemistry.

Results. Five- to 22-fold enhancement of paclitaxel cytotoxicity was achieved by paclitaxel + 17-AAG combination that was paralleled with marked induction of apoptosis. This combination treatment profoundly suppressed tumor growth and significantly prolonged survival of mice bearing H358 xenografts. Immunohistochemical staining of tumor tissues indicated profound reduction of vascular endothelial cell growth factor expression associated with reduction of microvasculature in tumors treated with 17-AAG. Apoptotic cells were more abundant in tumors treated with 17-AAG + paclitaxel than in those treated with 17-AAG or paclitaxel alone.

Conclusions. Concurrent exposure of H358 cells to 17-AAG and paclitaxel resulted in supraadditive growth inhibition effects in vitro and in vivo. Analysis of molecular markers of tumor tissues indicated that therapeutic drug levels could be achieved with this chemotherapy regimen leading to significant biological responses. Moreover, 17-AAG-mediated suppression of vascular endothelial cell growth factor production by tumor cells may contribute to the antitumor effects of this drug combination in vivo.

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Non-small cell lung cancers (NSCLC) frequently exhibit resistance to chemotherapy and ionizing radiation. Although dose-intensive regimens may increase response rates, they are frequently associated with severe systemic side effects. Treatment strategies designed to sensitize tumor cells to chemotherapeutic agents or radiation therapy may allow dose reduction, thereby diminishing systemic toxicity of cancer treatments.

The molecular basis of chemoresistance in cancer is complex and appears to involve both genetic and epigenetic factors, including overexpression of erbB1 and erbB2, encoding the epidermal growth factor receptor (EGFr) and the orphan receptor HER2/neu respectively. Inhibition of the function or the expression of the EGFr or HER2/neu sensitizes tumor cells, including those of NSCLC histology, to standard cytotoxic agents such as cisplatin or paclitaxel [1–3]. Previously we demonstrated that sensitivity to paclitaxel could be enhanced in NSCLC cells overexpressing erbB2 after treatment with 17-allylamino geldanamycin (17-AAG) [4]. This agent has been selected for clinical development at the National Cancer Institute because of its activity against cell lines derived from a variety of human malignancies [5, 6]; antitumor effects of this compound relate in part to its ability to inhibit the expression of various oncoproteins including EGFr and HER2/neu at nanomolar to micromolar concentrations [6, 7].

Overexpression of EGFr or HER2/neu oncoproteins in lung, esophageal, breast, and ovarian cancers correlates with locally advanced disease, distant metastases, and diminished survival in patients with these malignancies [8–10]. In vitro experimental data indicate that tumor cells overexpressing these oncoproteins exhibit one or more phenotypes associated with local invasion or dis-

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tant metastasis in vivo, including downregulation of E-cadherin expression, increased expression of matrix metalloproteinases, and vascular endothelial growth factor (VEGF), as well as accelerated invasion through artificial extracellular matrix [11–13]. Conceivably, inhibition of erbB-mediated signal transduction by monoclonal antibodies, which antagonize receptor ligand binding, or compounds such as 17-AAG, which deplete erbB1/erbB2 expression, may effectively reduce the metastatic potential of cancer cells. Exploiting the latter approach, we have recently demonstrated that treatment of NSCLC cells with 17-AAG at nanomolar concentrations for 48 hours results in profound suppression of VEGF and matrix metalloproteinase-9 secretion, as well as inhibition of cell motility through the artificial extracellular matrix membrane Matrigel (Sigma, St. Louis, MO) [14]. The relevance of these findings, particularly the antiangiogenic effects of 17-AAG-mediated suppression of VEGF production, have not been defined.

In the present study, we sought to examine the in vivo effects of paclitaxel and 17-AAG using nude mice bearing H358 NSCLC xenografts. Herein, we demonstrate that 17-AAG + paclitaxel treatment mediates profound retardation of tumor growth, and significant prolongation of survival in tumor-bearing animals. The in vitro effects of 17-AAG on H358 cells were closely reproduced in vivo: diminished erbB2 and VEGF expression in xenografts correlated with reduced tumor capillary density in animals receiving treatments containing 17-AAG. Apoptosis was most pronounced in xenografts from animals treated with 17-AAG + paclitaxel. Collectively these data support further evaluation of 17-AAG in combination with paclitaxel in lung cancer patients.

Material and Methods

Cells and Reagents

The NSCLC cell line H358 was purchased from American Tissue Culture Collection (Manassas, VA). Cells were maintained in RPMI media supplemented with glutamine (1 mmol/L), streptomycin (100 μg/mL)/penicillin (100 U/mL), and 10% of fetal calf serum. Normal human bronchial epithelial cells were purchased from Clonetics Corp (Walkersville, MD) and maintained in bronchial epithelial cell basal media (Clonetics Corp). The 17-AAG, obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutic Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, was dissolved in dimethylsulfoxide (DMSO) to yield a 100 μmol/L stock solution and stored at −70°C. The selective erbB2 tyrosine kinase inhibitor AG825 was obtained from Calbiochem/Oncogene Research Products (Cambridge, MA), dissolved in DMSO, and stored as 10 mmol/L stock solution. All experiments involving these two compounds were performed under subdued light conditions. Paclitaxel (Taxol, USP) was purchased from Bristol-Myers Squibb (Princeton, NJ). 4,5-Dimethylthiazo-2-yl-2,5-diphenyl tetrazolium bromide was purchased from Sigma (St. Louis, MO). Recombinant human epidermal growth factor (EGF) (R&D, Minneapolis, MN) and the anti-erbB1 and anti-erbB2 monoclonal antibodies (Calbiochem/Oncogene Research Products, Cambridge, MA) were constituted in phosphate-buffered saline and stored at 4°C as recommended by the manufacturers. Human VEGF enzyme-linked immunosorbent assay kit was obtained from R&D. A formulation of egg phospholipid emulsion in dextrose solution was used to dissolve 17-AAG for parenteral administration.

Immunofluorescent Staining and Flow Cytometric Analysis of erbB1 and erbB2 Expression

Surface expression of erbB1 and erbB2 on H358 or normal human bronchial epithelial cells was quantitated by flow cytometry using a Beckton-Dickinson fluorescence-activated cell sorter as described previously [14].

Quantitation of Vascular Endothelial Cell Growth Factor Production by H358 Cells

Cells were grown to 80% confluency in 12-well tissue culture plates, washed once with phosphate-buffered saline and media replenished with 1 mL of RPMI with 1% fetal calf serum with or without 20 ng/mL of EGF. In 17-AAG- or AG825-treated groups, appropriate aliquots of 17-AAG or AG825 stocks were added into the culture media to yield desired drug concentrations 2 hours before stimulating tumor cells with EGF. After 24 hours of incubation, conditioned media were harvested and frozen at −70°C. Cells from each well were collected and cellular protein was assayed by BCA technique (Pierce, Rockford, IL). The VEGF levels in the conditioned media were measured by enzyme-linked immunosorbent assay using a commercially available kit and expressed as picograms per milliliter per 24 hours per milligram of cellular protein.

In Vitro Evaluation of Drug Cytotoxicity

Cells were seeded in flat-bottom 96-well microtiter plates (4,000 cells/well). After an overnight incubation, cells were treated with either paclitaxel alone or 17-AAG + paclitaxel combination. Cells were exposed to various concentrations (ranging from 4 to 1,000 nmol/L) of paclitaxel for 90 minutes followed by 96 hours of further incubation in normal media or media containing 17-AAG (20 or 40 nmol/L). At the end of the incubation period, viable cells were quantitated by (4, 5-dimethylthiazo-2-yl)-2, 5-diphenyl tetrazolium bromide colorimetric assays as described by the manufacturer. Paclitaxel dose–response curves were plotted as a fraction of viable paclitaxel-treated cells relative to cells grown in normal media. The H358 cells treated with the 17-AAG + paclitaxel combination were plotted as fraction of viable cells relative to cells exposed to 17-AAG alone (to correct for the minor growth inhibitory effect of 17-AAG). Paclitaxel inhibitory concentration at 50% (IC_{50}) values for cells treated with paclitaxel alone or paclitaxel in combination with 17-AAG were derived from respective dose–response curves. A reduction of paclitaxel IC_{50} values in cells treated with the drug combination indicated increased cellular responsiveness to paclitaxel cytotoxic

effects mediated by 17-AAG. To further confirm the synergistic cytotoxic drug interaction effect of paclitaxel and 17-AAG, the combination index at 50% growth inhibition level (CI_{50}) was calculated [15]. The CI_{50} values less than 1, equal to 1, or more than 1 indicate synergistic, additive, or antagonistic cytotoxic drug interactions, respectively.

Apoptosis and Caspase 3 Activity

The H358 cells treated with paclitaxel alone (50 or 200 nmol/L) or paclitaxel with 17-AAG (20 nmol/L) were harvested at 48 and 60 hours after drug treatment for measurement of caspase 3 activity and apoptosis, respectively. Caspase 3 activity in cell lysates was measured by colorimetric assay (R&D). After normalization for total protein in the cell lysates, caspase 3 activity was expressed as fold increase over levels detected in untreated control cells. Apoptosis was quantitated by flow cytometry techniques using the Apo-BrdU kit (Pharmingen, San Diego, CA) and protocols contained therein.

In Vivo H358 Human Tumor Xenografts Model

The H358 human tumor xenografts were created in hind flanks of nude mice by inoculation of 10^7 cells suspended in 100 μL of phosphate-buffered saline. Palpable tumors of 170 to 200 mm^3 appeared approximately 4 weeks after tumor cell injection. Tumor-bearing animals then received either paclitaxel (1 mg/kg dissolved in 100 μL of phosphate-buffered saline once per week for 4 weeks), 17-AAG (10 or 25 mg/kg in 100 μL of carrier solution at three daily injections per week for 4 weeks) or paclitaxel + 17-AAG combinations (first doses of 17-AAG were administered concurrently with paclitaxel) by intraperitoneal injections. Control animals received phospholipid-based drug carrier solution alone. Orthogonal diameters of tumors were measured weekly. Animals were euthanized when tumors reached maximally allowable volumes of 2,500 to 2,800 mm^3. Tumor volumes were estimated using the following formula: $V = 0.52 \times a \times b \times c$, where a, b, c are orthogonal diameters.

Representative xenografts were harvested at the end of treatment for evaluation of cellular expression of VEGF, von Willebrand factor, erbB2, or activated caspase 3 using immunohistochemical techniques.

Data Analysis

Data were expressed as mean ± standard deviation of at least three independent experiments. Paired *t* test, Student's *t* test, and one-way analysis of variance (with Bonferroni test for pairwise comparisons) were performed for statistical analysis using Prism 2.0 software package (Graphpad Software, Inc, San Diego, CA).

Results

Depletion of erbB1 and erbB2 Expression on H358 Cells by 17-AAG

The H358 cells expressed high levels of erbB2 (approximately threefold higher than the level detected on normal human bronchial epithelial cells), with mean fluorescence intensity of 160 ± 11. Exposure of H358 cells to 20 or 80 nmol/L of 17-AAG for 24 hours resulted in a dose-dependent reduction of erbB2 mean fluorescence intensity to 80 ± 6 and 40 ± 8 ($p < 0.01$ versus baseline controls, n = 3) (Fig 1). Moreover, at the higher dose of 17-AAG, erbB2 expression was completely depleted in 65% of treated cells. In contrast, erbB1 expression in H358 cells was comparable to normal human bronchial epithelial cells, and depletion of erbB1 was achievable only at high concentration of 17-AAG. Exposure to 80 nmol/L of 17-AAG for 24 hours reduced erbB1 expression by 35% (mean fluorescence intensity of 110 ± 4 in treated cells versus 168 ± 12 in control cells, $p < 0.01$); all tumor cells remained positively stained for this receptor (Fig 1). Similar to a previously published report [16], 24-hour exposure of tumor cells to 17-AAG was more efficient in depleting erbB2 than erbB1 membrane expression. The 17-AAG also significantly inhibited H358 cell growth in vitro with the estimated IC_{50} value of 70 ± 5 nmol/L.

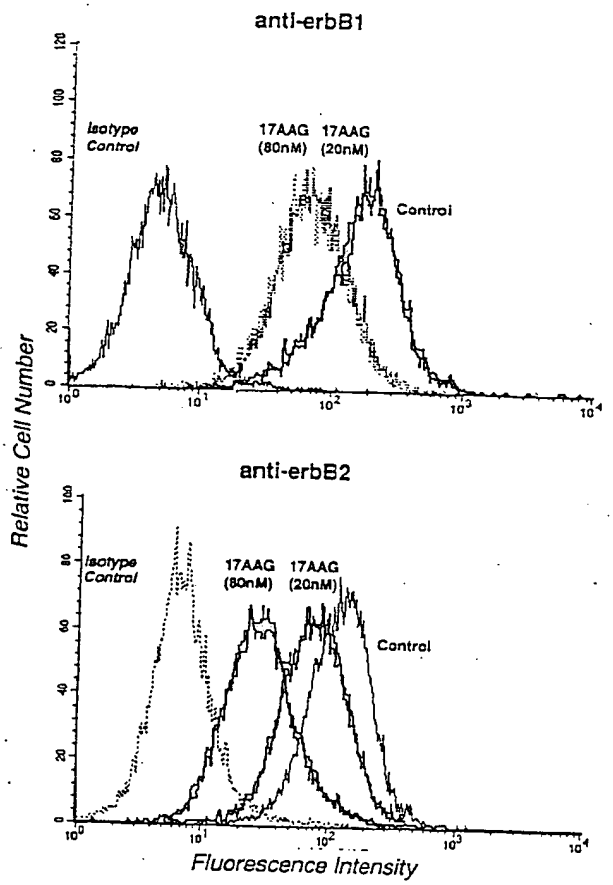

Fig 1. Immunofluorescence flow cytometric analysis of surface expression of erbB1 and erbB2 on H358 cells at baseline and after 24 hours of exposure to 17-allylamino geldanamycin (17-AAG) (20 or 80 nmol/L). Depletion of erbB1 (only at 80 nmol/L of 17-AAG) and erbB2 levels after 17-AAG treatment was indicated by significant reduction of erbB1 and erbB2 mean fluorescence intensity and shifting of the curves to the left. Representative data of three independent experiments that yielded similar results are shown.

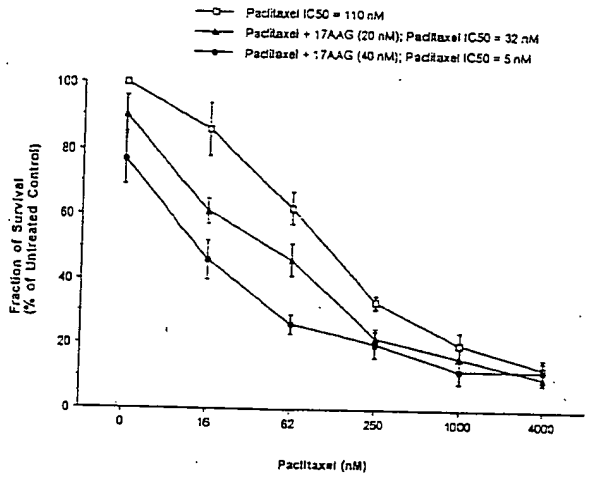

Fig 2. Enhancement of paclitaxel-mediated cytotoxicity in H358 cells by 17-allylamino geldanamycin (17-AAG) (20 or 40 nmol/L). The magnitude of growth inhibitory effect in cells treated with the drug combination was supraadditive, particularly at low doses of paclitaxel. Data are presented as mean ± standard deviation of four independent experiments, paclitaxel inhibitory concentration at 50% (IC50) values are indicated in the legends.

Enhancement of Paclitaxel Cytotoxicity by 17-AAG In Vitro

Figure 2 demonstrates the dose-dependent growth inhibitory effects of paclitaxel or paclitaxel + 17-AAG combination in H358 cells. Paclitaxel effectively inhibited tumor cell viability (IC_{50} = 110 ± 8 nmol/L). Concurrent exposure of H358 cells to paclitaxel and 17-AAG resulted in a drastic reduction of paclitaxel IC_{50} values (20 ± 3 and 5 ± 1 nmol/L at 20 nmol/L and 40 nmol/L of 17-AAG, respectively); these IC_{50} values were significantly lower than the paclitaxel IC_{50} value in cells treated with paclitaxel alone ($p < 0.001$). The increased suppression of cell growth by the addition of 17-AAG to paclitaxel treatment (30% to 40% at lower spectrum of paclitaxel dose range) was higher than the mild 17-AAG-mediated growth inhibition. This finding suggested that 17-AAG enhanced the susceptibility of tumor cell to the cytotoxic effect of paclitaxel. Furthermore, the synergistic cytotoxic effect of this drug combination was confirmed by the combination index CI_{50} values being significantly less than 1 (0.589 ± 0.078 and 0.630 ± 0.113 at 20 nmol/L and 40 nmol/L of 17-AAG, respectively, $p < 0.01$). The 17-AAG did not activate caspase 3 or induce apoptosis in H358 cells; in contrast, caspase 3 activity was evident in H358 cells after treatment with 17-AAG + paclitaxel, which was higher than that observed after exposure to paclitaxel alone (Fig 3A). This was accompanied by a higher magnitude of induction of apoptosis (Fig 3B). For instance, 55% ± 8% and 60% ± 4% of apoptotic cells were detected after treatment with 50 nmol/L and 200 nmol/L of paclitaxel in conjunction with 17-AAG compared to 22% ± 3% and 28% ± 4% of apoptotic cells after exposure to paclitaxel alone ($p < 0.01$).

Inhibition of Vascular Endothelial Cell Growth Factor Secretion From H358 Cells by 17-AAG and AG825 In Vitro

The H358 cells secreted high levels of VEGF into the culture media under basal conditions. Stimulation with EGF (20 ng/mL) resulted in a mild but significant elevation of VEGF (35,400 ± 3,750 versus 27,596 ± 2,500 pg/mL

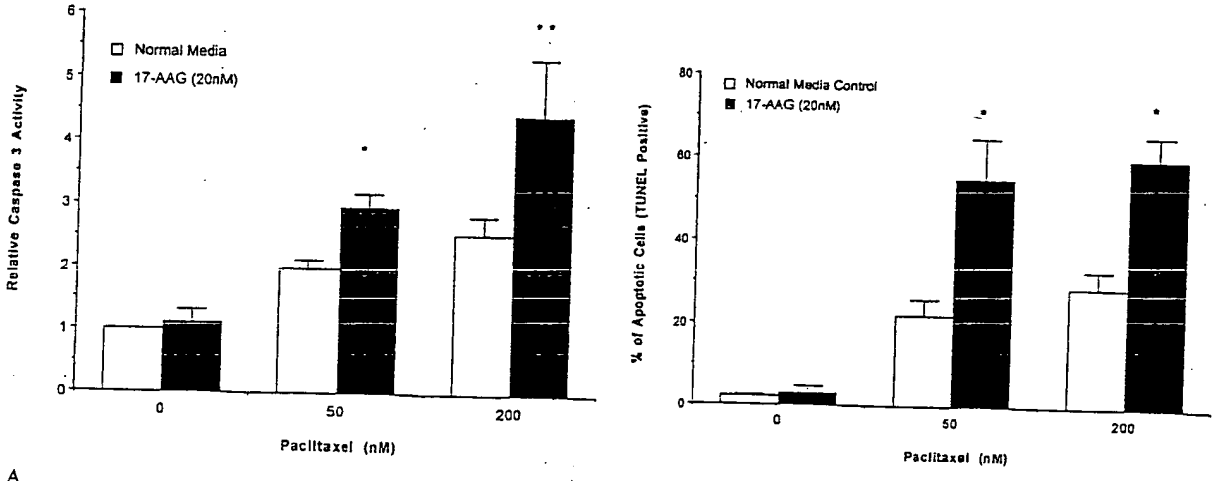

*Fig 3. (A) Caspase 3 activity in H358 cells treated with paclitaxel (50 or 200 nmol/L) alone or in combination with 17-allylamino geldanamycin (17-AAG) (20 nmol/L). Significantly higher levels of caspase 3 activity was observed in H358 cells treated with 17-AAG and paclitaxel combination than those treated with paclitaxel alone. Caspase 3 activity is expressed as fold of increase over baseline levels detected in control cells. Data are presented as mean ± standard deviation of four independent experiments, *$p < 0.01$, **$p < 0.001$. (B) Induction of apoptosis of H358 cells treated with paclitaxel (50 or 200 nmol/L) alone or in combination with 17-AAG (20 nmol/L). Apoptosis was quantitated in H358 cells harvested 60 hours after drug treatment by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay. Although 17-AAG at 20 nmol/L failed to induce apoptosis, combining 17-AAG with paclitaxel resulted in up to twofold increase in the induction of apoptosis. Data are presented as mean ± standard deviation of four independent experiments, *$p < 0.01$.*

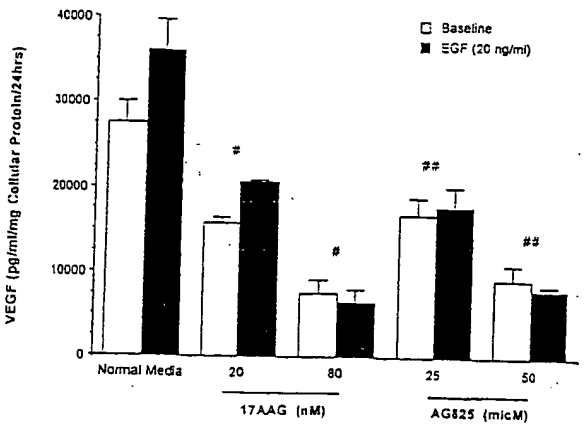

Fig 4. Levels of vascular endothelial cell growth factor (VEGF) in conditioned culture media of H358 cells at baseline or after stimulation with epidermal cell growth factor (EGF) (20 ng/mL) in the presence or absence of 17-allylamino geldanamycin (17-AAG) (20 or 80 nmol/L) or the selective erbB2 tyrosine kinase inhibitor AG825 (25 or 50 μmol/L). Not only did 17-AAG or AG825 strongly inhibit vascular endothelial cell growth factor production by H358 cells at baseline conditions but they also effectively abrogated the upregulation of vascular endothelial cell growth factor production by epidermal cell growth factor. Data are presented as mean ± standard deviation of four independent experiments. $^{\#}p < 0.01$, $^{\#\#}p < 0.001$.

per 24 hours per milligram of cellular protein, $p < 0.01$). Treating H358 cells with 17-AAG (20 or 80 nmol/L) for 24 hours suppressed both basal as well as EGF-mediated upregulation of VEGF expression (Fig 4) in a dose-dependent manner with the magnitude of inhibition ranging from 40% to 80%.

Inhibition of EGF-mediated upregulation of VEGF production by H358 cells after 17-AAG exposure may result from its effect on downregulating both erbB1 and erbB2 expression. The erbB2 receptor, although lacking a cognate ligand, modulates signaling from other members of the erbB superfamily [17]. To delineate further the role of erbB2 in EGF-mediated upregulation of VEGF production, additional experiments were performed using the selective erbB2 tyrosine kinase antagonist AG825 [18]. Treating H358 cells with AG825 (25 or 50 μmol/L) suppressed basal as well as EGF-mediated induction of VEGF secretion, to the same degree as observed after 17-AAG treatment (Fig 4).

In Vivo Tumoricidal Effect of Paclitaxel and 17-AAG

The in vivo experiment was designed to validate the in vitro findings of the synergistic cytotoxic effect and the potential antiangiogenic property of this combination (Fig 5). Parental administration of 17-AAG alone resulted in dose-dependent growth inhibition of H358 xenografts. Statistically significant reduction of tumor volumes and corresponding survival extensions were observed only in animals treated with the high dose of 17-AAG (25 mg/kg) (Fig 5 and Table 1). Significant inhibition of tumor growth and prolonged survivals were also noted in mice treated with paclitaxel (1 mg/kg) alone. More importantly, concurrent administration of 17-AAG (either at 10 or 25 mg/kg) and paclitaxel (1 mg/kg) resulted in a profound dose-dependent tumoricidal effects with 70% to 85% inhibition of tumor growth, and extension of survival from 3.5 months (low dose of 17-AAG with paclitaxel) to nearly 5 months (17-AAG 25 mg/kg and paclitaxel), representing a two- to threefold prolongation of life expectancy of tumor-bearing animals (Fig 5 and Table 1). The tumor volumes measured at 8 weeks after the beginning of combination chemotherapy were significantly less than those predicted by assuming additive drug effects (Table 1). Furthermore, the mean survival extensions of animals treated with 17-AAG + paclitaxel com-

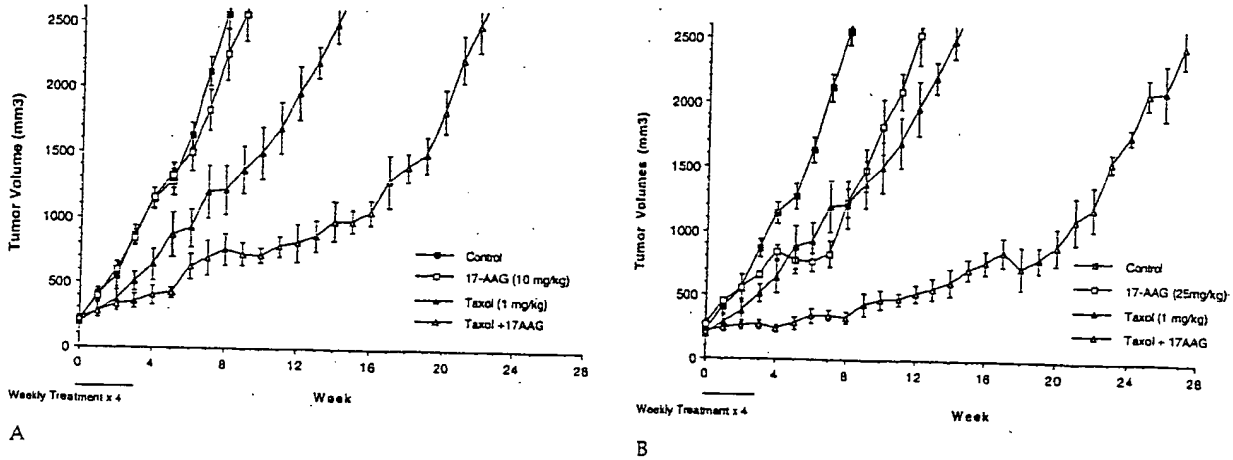

Fig 5. Enhancement of paclitaxel tumoricidal effect by 17-allylamino geldanamycin (17-AAG) in nude mice bearing H358 tumor xenografts. Animals were treated with either 10 mg/kg of 17-AAG (A) or 25 mg/kg of 17-AAG (B) in combination with paclitaxel (1 mg/kg) weekly for 4 weeks by intraperitoneal injections. Treatment schedule consisted of paclitaxel one injection/wk or 17-AAG three daily consecutive injections/wk for 4 weeks. Controls were animals treated with mock injection of the phospholipid-based carrier solution, with 17-AAG alone or with paclitaxel alone. Tumor dimensions were measured weekly until sacrifice. Data are presented as mean ± standard deviation, n = 8 per group. (Taxol = paclitaxel.)

Table 1. In Vivo Tumoricidal Effects of 17-AAG, Paclitaxel, and 17-AAG + Paclitaxel Combination

Group	Survival[a] (wk)	Mean Extension of Survival (wk)	Tumor Volumes at 8 Weeks (mm^3)
Control	7.8 ± 0.8	N/A	2,557 ± 110
17-AAG (10 mg/kg)	9.2 ± 1.6	1.4	2,370 ± 300
17-AAG (25 mg/kg)	13.5 ± 0.5[b]	5.7	1,077 ± 56
Paclitaxel (1 mg/kg)	14.3 ± 1.6[b]	6.5	1,210 ± 183
17-AAG (10) + paclitaxel	22.6 ± 1.6[b]	14.8	762 ± 120 (1174)[c]
17-AAG (25) + paclitaxel	26.8 ± 3.2[b]	19.0	328 ± 45 (508)[c]

Mean ± SD (n = 8 per group).

[a] Time elapsed from the onset of therapy to reaching tumor volumes of 2,500 mm^3. [b] $p < 0.001$ versus control by analysis of variance and Bonferroni test. [c] Predicted mean tumor volumes by assuming additive drug effects.

17-AAG = 17-allylamino geldanamycin.

binations were longer than the algebraic sums of the extensions resulting from each treatment alone (Table 1). Collectively, these data suggested in vivo synergistic tumoricidal drug effects.

Immunohistochemical analysis of erbB2 and VEGF expression, capillary density, and apoptosis in tumor nodules harvested 24 hours after completion of chemotherapy is shown in Figure 6. A profound reduction of membrane-bound erbB2 expression was observed in tumors from mice treated with either 17-AAG alone or 17-AAG + paclitaxel, suggesting that therapeutic levels of 17-AAG were achieved in xenografts. In parallel with in vitro observation of 17-AAG-mediated inhibition of VEGF production by H358 cells, cytoplasmic VEGF expression was also significantly decreased in tumors harvested from mice treated with 17-AAG or 17-AAG + paclitaxel (Table 2). Interestingly, mild-to-moderate reduction of cytoplasmic VEGF expression, was also noted in tumors treated with paclitaxel alone, similar to previously published observations [19]. Capillary density of tumor nodules was evaluated by staining for von Willebrand factor, which is expressed by capillary endothelial cells. Diffuse positive endothelial staining observed in control tumors was slightly diminished in tumors treated with paclitaxel. von Willebrand factor expression was markedly reduced in tumor treated with 17-AAG or 17-AAG + paclitaxel, indicating that reduction of VEGF expression coincided with diminished tumor angiogenesis (Table 2). Abundance of activated caspase 3-positive cells (indicative of ongoing apoptosis, dark brown staining cells) could be observed in tumors treated with the 17-AAG + paclitaxel drug combinations (Fig 6). Apoptotic cells were less frequently noted in paclitaxel-treated tumors, and rarely were they seen in control or 17-AAG-treated tumors.

Table 2. Semiquantitative Analysis of Immunohistochemical Staining of Tumor Tissues for Molecular Markers (n = 3)

Group	erbB2	VEGF	vW Factor	Activated Caspase 3
Control	++++	+++	+++	0
17-AAG (10 mg/kg)	++	++	++	0
17-AAG (25 mg/kg)	+	+	++	0
Paclitaxel (1 mg/kg)	+++	++	++	+
17-AAG (10) + paclitaxel	++	++	++	++
17-AAG (25) + paclitaxel	+	+	+	+++

17-AAG = 17-allylamino geldanamycin; VEGF = vascular endothelial cell growth factor; vW = von Willebrand.

Comment

Previous studies have demonstrated that members of the erbB superfamily (particularly erbB1 and erbB2, which are frequently overexpressed in NSCLC cells) play important roles in mediating the response of tumor cells to cytotoxic stress and modulating their susceptibility to chemotherapeutic agents. Overexpression of erbB2 in cancer cells enhances resistance to various cytotoxic agents including cisplatin and paclitaxel as well as irradiation. Upregulation of DNA repair mechanisms mediated by erbB2 overexpression contributes to chemotherapy and radiation resistance in cancer cells [3]. In contrast, upregulation of the cyclin-dependent kinase inhibitor p21, which inhibits progression of cells into the M phase of the cell cycle, is a major mechanism of paclitaxel resistance in cancer cells overexpressing erbB2 [20, 21]. Overexpression of erbB1 or activation of erbB1 receptor tyrosine kinase activity by EGF similarly renders tumor cells refractory to paclitaxel cytotoxicity [22]. Inhibition of the function or phenotypic expression of erbB1 or erbB2 by monoclonal antibodies or low molecular weight compounds abrogates chemoresistance mediated by erbB overexpression in cancer cells.

We have been interested in developing strategies to enhance the cytotoxic effects of conventional chemotherapeutic agents such as paclitaxel in lung or esophageal cancers by exploiting the current understanding of the relationship between the erbB signal transduction pathways and paclitaxel sensitivity. By targeting the erbB oncogenes using low molecular weight compounds such as 17-AAG we may avoid technical issues that may limit the efficacy of anti-erbB monoclonal antibodies, antisense constructs, or intracellular anti-HER2/neu single chain antibodies. Previously, we have demonstrated sequence-dependent enhancement of paclitaxel cytotoxicity in NSCLC by this compound [4]. Concurrent but not sequential 17-AAG/paclitaxel drug exposure significantly sensitized NSCLC cells expressing high levels of erbB2 to paclitaxel. This salutary effect has been extended to esophageal cancer cells expressing elevated levels of erbB2 (unpublished data). The in vivo experiments dem-

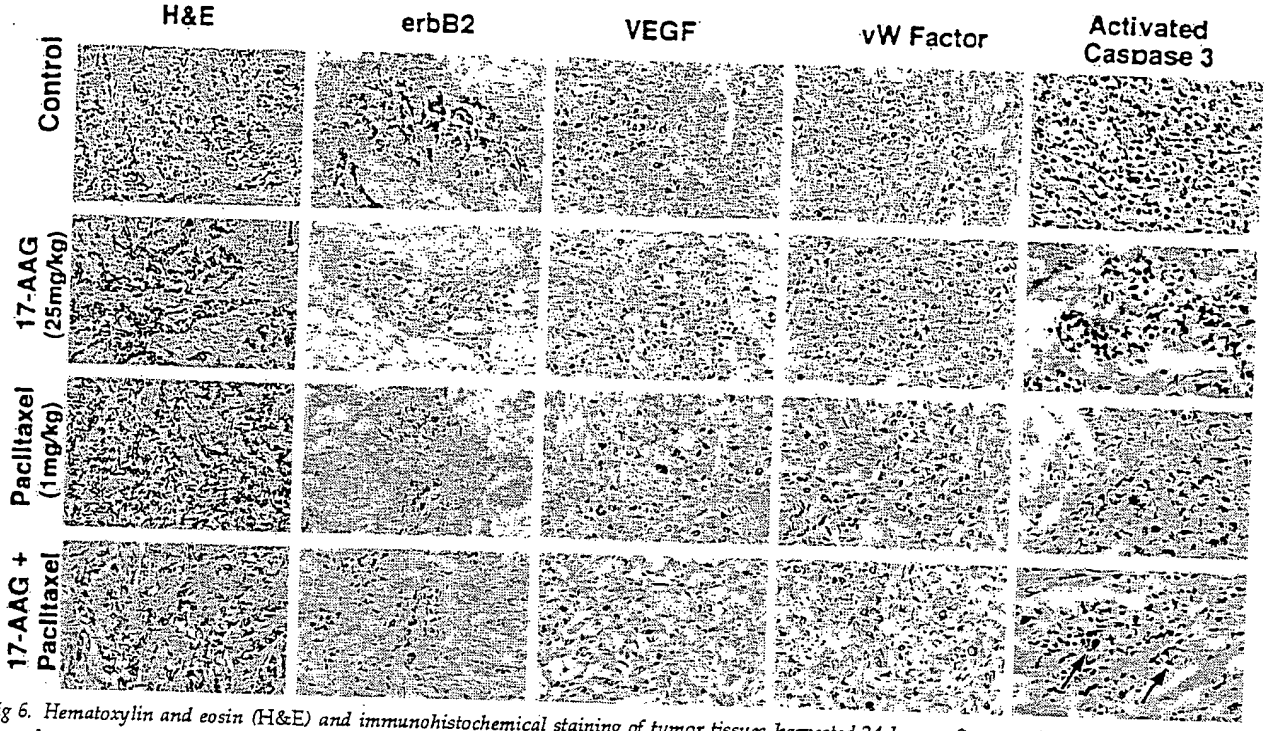

Fig 6. Hematoxylin and eosin (H&E) and immunohistochemical staining of tumor tissues harvested 24 hours after completion of the full 4-week course chemotherapy for molecular markers including erbB2, vascular endothelial cell growth factor (VEGF), von Willebrand (vW) factor (staining for endothelial cells to evaluate tumor microvasculature) and activated caspase 3 (only present in apoptotic cells). Representative photomicrographs of tumors treated with phosphate-buffered saline (control), 17-allylamino geldanamycin (17-AAG) (25 mg/kg), paclitaxel (1 mg/kg), or both are shown here. Hematoxylin and eosin: magnification, ×200; immunohistochemical staining: magnification, ×400.

onstrating profound tumoricidal effects of the paclitaxel and 17-AAG combination extended in vitro observations of synergistic cytotoxic drug interaction of these two agents. However, it is difficult to prove synergism of drug interactions in vivo. The observations that tumor volumes in animals treated by the drug combinations were smaller than predicted by assuming additive antitumor drug effects (Table 1) and the survivals of tumor-bearing animals treated with the drug combination were longer than the algebraic sums of the extension of survivals achieved by individual drug treatment alone strongly support the notion of potentiation of paclitaxel tumoricidal effect by 17-AAG.

In addition to being a chemotherapy sensitizer, our studies have shown that 17-AAG is a powerful inhibitor of VEGF production by tumor cells through its negative effect on erbB signal transduction pathways, which are known to transcriptionally regulate VEGF gene expression [13, 23]. Significant information was derived from the molecular analysis of tumor samples harvested after completion of a 4-week course of chemotherapy. This time point was chosen to clearly demonstrate the antiangiogeneic effect of 17-AAG. Whereas depletion of VEGF secretion by H358 cells occurs within hours of 17-AAG exposure, alteration of tumor microvasculature in response to reduced VEGF levels would take more time to develop. The in vivo effects of 17-AAG on erbB2 or VEGF expression and the induction of apoptosis by paclitaxel (with or without 17-AAG) were probably derived from the last treatment rather than a cumulative effect of the four cycles. Almost complete depletion of erbB2 expression in tumors treated with regimens containing 17-AAG provided direct proof that therapeutic drug levels were achieved using the treatment schedule discussed. In conjunction with depletion of erbB2 expression, significant reduction of VEGF cytoplasmic expression in H358 tumor cells and inhibition of angiogenesis were also observed. These data clearly demonstrate the biological significance of 17-AAG-mediated inhibition of VEGF production, and affirm our in vitro experiments that suggested that 17-AAG exhibits antiangiogenic effects. In this context, 17-AAG played the dual role of a paclitaxel-sensitizing and an antiangiogenic agent. This drug combination strategy appears quite analogous to recent anticancer regimens combining a cytotoxic agent and an antiangiogenic agent (either an anti-VEGF antibody or a VEGF receptor pharmacologic antagonist) [24, 25]. The individual contribution of the paclitaxel sensitization and the antiangiogenic properties of 17-AAG are difficult to define at this time.

The phase I clinical trial using 17-AAG (daily 1-hour intravenous infusion for 5 days every 3 weeks) as a single agent to treat advanced solid malignancies is in progress at the National Cancer Institute, and initial data indicate that hepatotoxicity is the dose-limiting factor as predicted from preclinical studies (Jean Grem and Richard

Wilson, National Cancer Institute, Bethesda, MD, personal communication). The peak plasma drug levels at maximally tolerated dose were 1 to 2 μmol/L, which are well above the concentrations required to achieve synergistic drug interaction in vitro. The acceptable drug toxicity profile derived from the phase I trial together with the powerful tumoricidal effect of this drug combination observed in our present studies support evaluation of 17-AAG in combination with paclitaxel in patients with NSCLC and esophageal cancers exhibiting high level HER2/neu expression.

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DISCUSSION

DR HARVEY I. PASS (Detroit, MI): Very nice, Dr Nguyen.

I have a couple of questions. The relationship between erbB-2 and this whole situation, what is the evidence that AAG is actually working selectively through an erbB-2 mechanism and is not having other effects, and have you done experiments to block either with antibody or making it even better using Herceptin (Genentech, San Francisco, CA)?

The second question is, this could also be explained by overcoming resistance to paclitaxel. Do you have any data with regard to your cell line with regard to β tubulin mutations in this cell line?

DR NGUYEN: To answer your first question, in terms of the impact of 17-AAG on erbB-2-mediated signal transduction path-

way, you are perfectly right. This compound not only targets erbB-2, it also targets other signal transduction pathways as well as other oncoproteins. To specifically target erbB-2 pathway, we used Herceptin and we also used AG825, which is a selective erbB-2 antagonist. We observed similar enhancement of paclitaxel-mediated cytotoxicity in NSChC cells by AG825. Herceptin, on the other hand, we did not see such a powerful sensitization effect, and the reason for that is unclear at this point.

What is your second question, Dr Pass?

DR PASS: The question of whether this is simply an overcoming chemoresistance phenomenon. Do you have any data whether AAG has any effect on cell lines with β tubulin mutations, and does your cell line have β tubulin mutation?

DR NGUYEN: No, we have not done that. We recently looked at the mechanisms of this combination effect, and it is related to the ability of 17-AAG to suppress p21, and that allowed the cells to traverse the G2M check point and accumulate the cells in mitosis, and p21 actually inhibits cdC2/p34 kinase, which is known to play an important role in paclitaxel-mediated apoptosis. So we observed that by using this combination the p21 levels decreased, associated with higher fraction of cells in mitosis and augmentation of paclitaxel-mediated apoptosis.

DR PASS: Do you have any data with any other chemotoxics?

DR NGUYEN: It has been looked at before with 17-AAG-sensitized tumor cells to cisplatin and the other chemotherapeutics.

Notice From the American Board of Thoracic Surgery

The 2001 Part I (written) examination will be held at the Hotel Sofiel, 5500 North River Rd, Rosemont, IL 60018-5194, on November 18, 2001. The closing date for registration was August 1, 2001.

To be admissible to the part II (oral) examination, a candidate must have successfully completed the part I (written) examination.

A candidate applying for admission to the certifying examination must fulfill all the requirements of the Board in force at the time the application is received.

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